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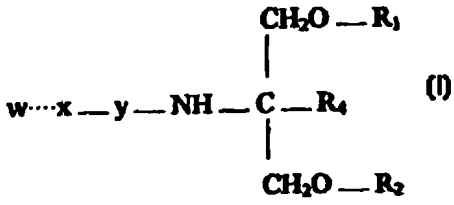

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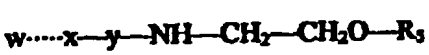

(II)

(57) Abstract

The present invention provides a method for introducing nucleic acids into cells. The method involves exposing the cells to a compound having formula (I) in which: w is a nucleic acid, x is a non-amino acid or non-peptide nucleic acid binding group, y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent, R_4 is H or halogen or CH_2O - R_3; and R_1, R_2 and R_3 are the same or different and are either hydrogen, methyl, ethyl, alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl groups or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl groups, optionally being an acyl group derived from a fatty acid having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that at least one of R_1, R_2 or R_3 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated, or to a compound having formula (II) in which: w is a nucleic acid, x is a non-amino acid or non-peptide nucleic acid binding group, y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent, R_5 is alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl group or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl group optionally being an acyl group having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that R_5 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated. The invention also relates to these compounds.

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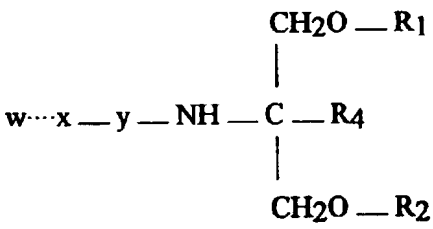
Composition and Method for Delivery of Nucleic Acids

The present invention relates to a method of introducing compounds, in particular nucleic acids, into cells. Further, the present invention relates to compositions for use in this method.

There are a number of situations in which it is desirable to deliver specific compounds into cells. One of these applications is the transfection of eucaryotic cells with DNA. This is currently done using various commercial lipofecting agents such as "Transfectam" (Promega) and "Lipofectin" (BRL), by using calcium phosphate mediated transfection or by electroporation of cells.

The ability to deliver nucleic acid based compounds to cells also has application in drug delivery. Delivery of drugs into cells or tissues in association with a compound of the formula described below will change parameters such as the duration of drug action (eg. slow release or sustained action), the amount of drug required or the mode of delivery. The delivery of drugs using compounds variant within the parameters described below should also enable more specific targeting of drug delivery both within cells and whole organisms.

As disclosed in the present applicants' co-pending International patent application No. PCT/AU95/00505 (the disclosure of which is incorporated herein by cross-reference), nucleic acid may be introduced into a cell by exposing the cell to a compound of the formula:



in which:

w is a nucleic acid

x is an amino acid or peptide

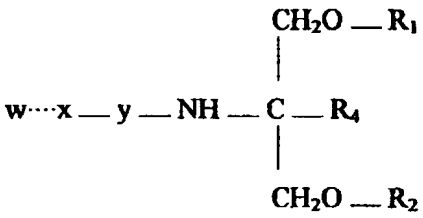
y is a spacer having a chain length equivalent to 1-20 carbon atoms or is absent

R_4 is H or CH_2O-R_3; and R_1, R_2 and R_3 are the same or different and are either hydrogen, methyl, ethyl, hydroxyl or acyl groups derived from a fatty acid having

a carbon chain of 3 - 24 carbon atoms saturated or unsaturated with the proviso that at least one of R_1, R_2 and R_3 is an acyl group derived from a fatty acid.

The present inventors have now shown that it is possible to introduce nucleic acid into a cell using similar compounds but in which the nature of "x" is varied.

Accordingly, in the first aspect the present invention consists in the method of introducing nucleic acid into a cell comprising exposing the cell to a compound having the formula:



in which:

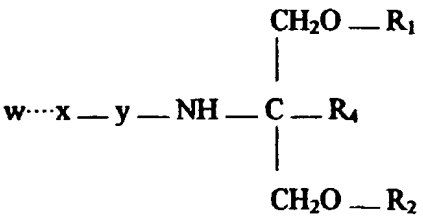
w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_4 is H or halogen or CH_2O - R_3; and R_1, R_2 and R_3 are the same or different and are either hydrogen, methyl, ethyl, alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl groups or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl groups, optionally being an acyl group derived from a fatty acid having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that at least one of R_1, R_2 or R_3 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

In a second aspect the present invention consists of a compound for the use of introducing nucleic acid into a cell, the compound having the formula:



in which:

w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_4 is H or halogen or CH_2O - R_3; and R_1, R_2 and R_3 are the same or different and are either hydrogen, methyl, ethyl, alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl groups or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl groups, optionally being an acyl group derived from a fatty acid having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that at least one of R_1, R_2 or R_3 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

As used herein the term "nucleic acid" is used in its brodest sense and includes DNA, RNA or oligonucleotides of either DNA or RNA, modified oligonucleotides and combinations of these. In addition, the term is intended to cover modified oligonucleotides. These modifications include, but are not limited to, sugar modificatons, such as 2'-deoxy-2'-fluoro nucleotides (Kawasaki, A.M. et al.(1993) J. Med. Chem **36**: 831-834), 2'-O-allyl-uridine, -cytosine, -adenosine and -guanosine (Beijer, B *et al.* (1994) Nucleos. Nucleot. **13**: 1905-1927), 2'-deoxy-2'-C-allyl-ribonucleotides, 2'-O-methyl ribonucleotides (Sproat, B.S. and Lamond, A.I. (1991) in "Oligonucleotides and analogues; a practical approach" pp 49-86, F. Eckstein (ed.). Oxford University Press, Oxford) and other 2'-O-alkyl ribonucleotides (Sproat, B.S. *et al.* (1991) Nucleic Acids Symp. Ser., 24: 59-62), phosphate modifications, such as phosphorothioates (Stec, W.J. *et al.* (1991) Nucl. Acids Res., **19**: 5883-5888), phosphordithioates (Beaton G. *et al.* in "Oligonucleotides and analogues; a practical approach" pp 109-136 F. Eckstein (ed.). Oxford University Press, Oxford), phosphoramidates (Froehler, B., Ng, P. and Matteucci, M.D. (1998) Nucl. Acids Res. **16**: 4831-4839), methyl phophonates (Miller, P.S. *et al.* (1986) Biochemistry **25**: 5092-5097) and other aklyl-phosphonates (Fathi, R. *et al.*(1994) Nucl. Acids Res. **22**: 5416-5424), and other backbone modifications such as amides (including but not limited to PNA) (De Mesmaeker, A. *et al.* (1994) Agnew Chem. Int. Ed. **33**: 226-229; Egholm, M. *et al.* (1992) J. Am. Chem. Soc. **114**: 1895-1897) , carbamate (Habus, I, Temsamani, J. and Agrawal, S. (1994) Bioorg. Med. Chem. Lett. **4**:1065-1070), Ureas (Waldner, A. *et al.* (1994) Synlett. 57-61), amines (Caulfield, T.J. et al. (1994) Bioorg. Med. Chem. Lett. **3**: 2771-2776), allyl ether (Cao, X. and Matteucci, M.D. (1994) Tet. Lett. **35**: 2325-2328), allyl (Cao, X. and Matteucci, M.D. (1994) Tet. Lett. **35**: 2325-2328). Alkane (De Mesmaeker, A. *et al.* (1994) Bioorg. Med. Chem. Lett. **4**: 395-

398), morpholino (Stirchak, E.P., Summerton, J.E. and Weller, D.D. (1989) Nucl. acids Res. 17: 6129-6141) and guanidinium (Dempcy, R.O., Almarsson and Bruice, T.C. (1994) Proc. Natl. Acad. Sci. (USA) 91: 7864-7868) (produces a polycationic oligonucleotide). In addition, the oligonucleotides may contain base modifications, terminal and internal modifications adding functional groups such as psoralen, biotin or cholesterol groups and non-nucleotide regions.

The nucleic acid "w" may associate with the non-amino acid or non-peptide nucleic acid binding group in any manner such as covalent bonding, ionic interaction, hydrogen bonding, base pairing etc. As will be readily apparent to those skilled in this field the particular nature of the non-amino acid or non-peptide nucleic acid binding group "x" used will depend on the paticular nature of the nucleic acid "w" to be delivered. Where the association between "w" and "x" is an ionic interaction clearly where the nucleic has an overall positive charge the binding binding group "x" will have an overall negative charge.

It is currently preferred, however, that the association between "w" and "x" is an ionic interaction or hydrogen bonding or triplex formation.

Where the nucleic acid "w" is negatively charged the nucleic acid binding group "x" may be a cation or polycation of any length. In this embodiment, the nucleic acid binding group will typically possess an overall positive charge to attract and hold the nucleic acid by ionic interaction. For example, the nucleic acid binding group may be a polyamine such as spermine, a polyimine or a dendrimer.

The nucleic acid binding group "x" or the spacer "y" may also include functional domains that facilitate cellular targeting or subcellular localisation eg. sugars, receptor ligands, polysaccharides or peptides (such as nuclear localisation signals, antibodies or derivatives thereof eg. FABs or SCFVs). Such functional domains may be included in tandem or on a bifurcating structure, as homo or heterodimers.

In another embodiment of the present invention the nucleic acid binding group "x" is itself an oligonucleotide of either DNA or RNA, a modified oligonucleotide or a combination of these or a molecule of PNA capable of binding the nucleic acid "w" through base pairing, triple helix formation or similar interactions.

Nucleotides are the building blocks of nucleic acids. In their most abundant natural forms, they consist of a pentose sugar (ribose or 2-deoxyribose) with a hydrophobic nitrogenous base (a purine, Adenine or Guanine, or a pyrimidine, Thymine (Uracil in ribonucleotides) or Cytosine) covalently attached to the 1' carbon and phosphoric acid esterified to the free hydroxyl group on the 5' carbon of the pentose sugar. Nucleic acids are polymers of successive nucleotide units in which one

nucleotide is linked to the next by a phosphodiester bridge between the 3'-hydroxyl group of the pentose moiety of one nucleotide and the 5'-hydroxyl of the pentose moiety of the next. While there are two main sugars found in nucleic acids, in nature any particular nucleic acid does not contain both of these at the same time. Thus there are two kinds of nucleic acid, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Furthermore, the covalent backbone of a nucleic acid consist of alternating pentose and phosphoric acid groups and provides a molecular scaffold with a defined polarity from which the side-chain purine and pyrimidine bases are displayed. It is the sequence of the bases as they occur along this polarised polymer that endows nucleic acids with their special properties.

The property of nucleic acids (or more specifically the individual bases) that is crucial to this embodiment of the present invention is that the bases can pair with each other through hydrogen bonding. In general Adenine base pairs with Thymine (Uracil in RNA) and Guanine with Cytosine (complementary base pairing). Where base pairing occurs between bases on different nucleic acid molecules, the appropriate interactions between bases occur if the polarity of the sugar/phosphate backbones from which the bases are displayed are in opposite orientations in the two strands. The hydrogen bonding that occurs in these base interactions provides a force that can potentiate and stabilise associations between different nucleic acid molecules. The strength of these associations depends upon the degree of complementarity of the base sequences of the interacting strands when they are aligned with opposite polarities. In an aqueous solvent, complementary strands of nucleic acid associate to form a double helix or duplex with the sugar phosphate backbone in contact with the solvent and the hydrophobic bases stacking in the middle of the helix. Thus, in addition to the force of hydrogen bonding between all the composite base pairs, the London dispersion forces and hydrophobic effects caused by interactions between the stacked bases further serve to stabilise the association between complementary strands of nucleic acid. (Saenger, W. (1984) Principles of nucleic acid structure. Springer-Verlag (New York). While Adenine-Thymine (Uracil) and Guanine-Cytosine base pairs are the most common in nature, other base pairings can occur and can still contribute significantly to stabilisation of a duplex (Saenger (1984) ibid.).

The development of chemical methods for the synthesis of nucleic acids has made it possible to produce chimaeric DNA/RNA molecules. In addition it has provided the means of synthesising nucleic acids with altered bases and sugars and phosphate groups which can often lead to the improved stability of these molecules in biological fluids (see "Oligonucleotides and analogues, a practical approach" (1991)

IRL Press at Oxford University Press, Oxford, Eckstein F. ed.). In addition modified amino acids, in which amino acid side chains are replaced by the purine and pyrimidine bases found in nucleic acids, can also be synthesised (Egholm, M., Buchardt, O., Nielsen, P.E. and Berg, R.H. (1992). J. Am. Chem. Soc. **114** 1895-1897; Egholm, M., P. E. Nielsen, O. Buchardt and R. H. Berg. (1992). J. Am. Chem. Soc. **114**, 9677-9678). These protein-nucleic acid molecules or PNA can also associate with a nucleic acid of complementary sequence through base pairing (Nielsen, P. E., Egholm, M., Berg, R. H. and Buchardt, O, (1993). Anticancer Drug Des. **8**, 53-63; Hanvey, J. C., Peffer, N. J., Bisi, J. E., Thomson, S. A., Cadilla, R., Josey, J. A., Ricca, D. J., Hassman, C. F., Bonham, M. A., Au, K. G. et al. (1992). Science. **258**, 1481-5; Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B. and Nielsen, P. E. (1993). Nature. **365** 566-8; Nielsen, P. E., Egholm, M., Berg, R. H. and Buchardt, O. (1993). Nucleic Acids Res. **21**, 197-200).

While the interactions between the nucleic acid, modified nucleic acid and PNA species described above all involve interactions between two molecules, nucleic acids consisting of homo pyrimidine tracts can associate with double stranded nucleic acid carrying a region of duplex comprising complementary sequences of homo purine and the same homo pyrimidine tract. In such a ternary complex, the homo purine homo pyrimidine tracts of the initial duplex remain antiparallel and Watson-Crick base paired in the normal manner, while the invading homo pyrimidine strand fits into the deep major groove of this double helix and is involved in Hoogsteen-type base pairing with the bases of the polypurine containing strand. In such triple helical structures, the polarity of the invading polypyrimidine strand is the same as that of the polypurine strand (In Saenger, W. (1984) Principles of nucleic acid structure. Springer-Verlag (New York). The nucleic acids contributing to the triplex can be RNA or DNA although there is some evidence to suggest that an RNA third strand may bind more strongly to a DNA/DNA duplex than the corresponding DNA third strand (Roberts, R.W. and Crothers, D.M. (1992) Science **258**: 1463-1466). Modified oligonucleotides may also be used as the third strand eg 4'-thio-RNA (Leydier C., Bellon, L. Barascut, J-L., Morvan, F., Rayner, B. and Imbach, J-L.(1995) Antisense Res. and Dev. 5: 167-174). Triplexes have also been formed in which the third strand is a polypurine-containing nucleic acid (Porumb, H., Dagneaux, C., Letellier, R., Malvy, C. and Taillandier, E. (1994) Gene **1494**: 101-107). In this example the complex was based on reverse Hoogsteen G(GC) and A(AT) triplets (the pairs in parenthesis indicating the

Watson-Crick base pairs present in the initial duplex) with anti orientations of the bases and all the strands having S-type sugar conformations.

From the forgoing discussion it will be apparent that base pairing in a variety of forms can facilitate the association of two molecules of nucleic acid or a molecule of a nucleic acid and another of PNA or, in special circumstances, a single stranded nucleic acid with a nucleic acid duplex to form a triple helical structure. In all cases, it is well known that binding affinities are dependent on the length of homology, the level of complementarity, the base composition of the interacting sequences and the pH and ionic strength of the solution (for double stranded interactions see Britten, R.J and Davidson, E.H. (1985) in "Nucleic Acid Hybridisation: a Practical Approach", IRL Press Oxford. Washington D.C., Hames, B.D and Higgins, S.J. eds; Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press, Nolan, C. ed.: for triplexes see Rougee, M., Faucon, B., Mergny, J.L., Barcelo, F., Giovannangeli, C., Garestier, T. and Helene, C. (1992) Biochemistry **31**: 9269-9278; Singleton, S.F. and Dervan, P.B. (1992) Biochemistry **31**: 10995-11003; Singleton, S.F. and Dervan, P.B. (1992) J. Am. Chem. Soc. **114**: 6957-6965: for nucleic acid/PNA see Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B. and Nielsen, P. E. (1993). Nature. **365**: 566-8; Egholm, M., Buchardt, O., Nielsen, P. E. and Berg, R. H. (1992). J. Am. Chem. Soc. **114**: 1895-1897). Thus in the current invention, a single stranded nucleic acid or PNA "x" group with defined sequence properties could function as a nucleic acid binding domain in the compound either by base pairing to a complementary single stranded region on or by forming a triplex with a suitable double stranded sequence in the nucleic acid to be delivered.

It will be apparent to those skilled in the art that transfection reagents that bind the molecule to be delivered through base pairing would have significant advantages over reagents in which binding occurs via charge interactions for facilitating the delivery of nucleic acid targetted therapeutic agents with uncharged backbones (such as PNA and uncharged oligonucleotide analogues) into cells.

In an alternative embodiment, for example, where the nucleic acid (w) to be delivered is an oligonucleotide it could be covalently linked to the x group, particularly where the x-linker-lipophilic complex is a phosphoramidite.

Another example of nucleic acid binding groups are polyamides as exemplified by the antibiotics distamycin and netropsin. The repeating amide of distamycin is formed from an aromatic carboxylic acid and an aromatic amine combined to form a crescent shaped tripeptide. This tripeptide binds in the minor groove of double stranded

DNA at sites of five successive A·T base pairs but shows no appreciable binding to single stranded DNA or RNA. Binding of the antibiotic to the double stranded DNA is believed to be stabilised through a combination of hydrogen bonding to the base pairs, van der Waal's contacts and electrostatic forces (Coll, M., Frederick, C.A., Wang A.H. and Rich, A (1987) Proc. Natl. Acad. Sci. USA 84: 8385-8389). Although both netropsin and distamycin bind as monomers in the minor groove, at high concentration distamycin can bind as a dimer (Pelton, J.G. and Wemmer, D.E. (1989) Proc. Natl. Acad. Sci. USA 86: 5723-5727). Based on these observations, structurally related polyamides containing *N*-methylimidazole and *N*-methylpyrrole amino acids have been synthesised and shown to combine as antiparallel side-by-side dimeric complexes with the minor groove of DNA (Mrksich, M., Wade, W.S., Dwyer, T.J., Geierstranger, B.H., Wemmer, D.E. and Dervan P.B. (1992) Proc. Natl. Acad. Sci. USA 89: 7586-7590). Covalently linking such polyamide heterodimers and homodimers has led to the design of ligands with both increased affinity and specificity (Mrksich, M., Parks, M.E. and Dervan, P.B (1994) J. Am. Chem. Soc. 116: 7983). Such hairpin linked polyamides can now be synthesised efficiently and in large scale using solid phase synthesis (Baird, E.E and Dervan, P.B. (1996) J. Am. Chem. Soc. 118: 6141-6146) and carry reactive terminal groups that could be readily coupled either directly or via a spacer group to a lipophilic domain of the current invention.

Other potential "x" groups capable of complexing with nucleic acids by the formation of hydrogen bonds other than those occurring in base pairing are exemplified by polyvinyl derivatives such as polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP). PVA and PVP are both capable of forming hydrogen bonds, acting as hydrogen-bond donors and acceptors respectively (Galaev, Y, Garg, N. and Mattiasson, (1994) J. Chromatogr. A. **684**: 45-64; Buhler, V., (1993) Kollidon: Polyvinyl pyrrolidone for the pharmaceutical industry. BASF Aktiengellschaft Feinchemie, Ludwigshafen). PVP in particular has been shown to interact with DNA and protect it from nuclease attack while both PVP and PVA have been shown to enhance DNA uptake by rat skeletal muscle (Mumper, R.J., Duguid, J.G, Anwer, K., Barron, M.K., Nitta, H. and Rolland, A.P. (1996) Pharma. Res. 13:701-709). Shorter oligomers of vinyl alcohol and vinyl pyrrolidone, when coupled via a spacer moiety to a lipophilic domain, could bind nucleic acid and facilitate its uptake by cells. PVA is often produced by the hydrolysis of polyvinyl acetate with alkali. While alcohol groups of PVA or oligo VA are relatively unreactive, linkage could be achieved through reactive acetate groups on partially hydrolysed oligo VA. Using a similar rationale if PVP (or oligo VP) were produced by reacting a compound such as polyvinyl chloride (or

oligovinyl chloride) with pyrrolidone, coupling to a spacer group in the current invention could be achieved through residual chloride groups on incompletely derivatized PVP (or oligo VP) product.

The spacer "y" joining the nucleic acid binding group "x" to the amino group of the linker, for example, Tris may be any of a number of molecules well known in the art. The nature of the spacer will depend on the reactive groups available on the nucleic acid binding moiety "x". Examples of spacers "y" are:

1. Between an "x" group with an amino group and the amino group of the linker.

a) a spacer with a carboxyl group to the "x" moiety and a carboxyl group to the amino group, such as dicarboxylic acids via the anhydride, eg. succinic anhydride, maleic anhydride etc.

b) a spacer with an carboxyl group to the "x" moiety and an aldehyde group to the amino group such as glyoxylic acid (in the presence of reducing agent, eg. Na BH_4) or vice versa.

c) a spacer with a carboxyl group to the "x" moiety and a halide to the amino group such as chloroacetic acid or vice versa.

d) a spacer with a halide group to "x" and a sulphonic acid group to the amino group such as 2-bromoethanesulphonic acid or vice verse.

e) a spacer with a chloroformate group to "x" and a halide group to the amino group such as bromoethyl chloroformate or vice versa.

f) a spacer with a halide group to "x" and a halide group to the amino group such as 1,2 dibromoethane

g) other examples of potentially suitable spacers between an "x" group with an amide group and the amino group include the compound families exemplified by acrylic acid.

2. Between an "x" group with an hydroxyl group and the amino group of the linker.

a) a spacer with a carboxyl group to the "x" group and a carboxyl group to the amino group, such as dicarboxylic acids via the anhydride, eg. succinic anhydride, maleic anhydride etc.

b) a spacer with an carboxyl group to the "x" moiety and an aldehyde group to the amino group such as glyoxylic acid (in the presence of reducing agent, eg. $NaBH_4$).

c) a spacer with a carboxyl group to the "x" moiety and a halide to the amino group such as chloroacetic acid.

d) a spacer with a carboxyl group to the "x" moiety and an N=C=O group to the amino group such as ethylisocyanatoacetate.

3. Between an "x" group with a thiol group and the amino group of the linker.

a) a spacer with a carboxyl group to the "x" group and a carboxyl group to the amino group, such as dicarboxylic acids via the anhydride, eg. succinic anhydride, maleic anhydride etc.

b) a spacer with an carboxyl group to the "x" moiety and an aldehyde group to the amino group such as glyoxylic acid (in the presence of reducing agent, eg. $NaBH_4$).

c) a spacer with a carboxyl group to the "x" moiety and a halide to the amino group such as chloroacetic acid.

d) a spacer with a carboxyl group to the "x" moiety and an N=C=O group to the amino group such as ethylisocyanatoacetate.

4. Between an "x" group with a free carboxyl group and the amino group of the linker.

a) a spacer with an amino group to the "x" moiety and a carboxyl group to the amino group eg. an amino acid or an antibiotic.

b) a spacer with an amino to the "x" moiety and a sulphonic acid group to the amino group eg. 2-aminoethanesulphonic acid (taurine).

c) a spacer with an hydroxyl to the "x" moiety and a carboxyl group to the amino group eg. glycolic acid, lactic acid etc.

d) a spacer with an hydroxyl group to the "x" and a sulphonic acid group to the amino group eg. 2-hydroxyethanesulphonic acid.

e) a spacer with an hydroxyl group to the "x" moiety and a reactive halide to the amino group eg. 2-chloroethanol.

f) other examples of potentially suitable spacers between a compound with a reactive carboxyl and the amino group include compound families exemplified by p-hydroxybenzaldehyde, 2-chloroacetic acid, 1,2-dibromomethane and ethyleneoxide.

As is clear from the above description the spacer "y" may be absent, however, it is preferred that the molecule does include spacer "y". As stated above the spacer may be any of a number of molecules well known in the art. It is, however, presently preferred that the spacer has a chain length equivalent to 3 to 17 carbon atoms. In this regard it is particularly preferred that the spacer is amino butyric, amino caproic, amino caprylic, amino undecanoic acid or a dipeptide of amino caproic acid and amino undecanoic acid.

In a preferred embodiment of the present invention R_1, R_2 and R_3 are alkyl, hydroxy alkyl or alkenyl groups. Preferred alkyl groups are derived from 1-bromo

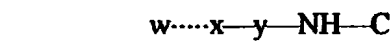
undecane, 1-bromotridecane or 1-bromo hexadecane. Preferred hydroxy alkyl groups are derived from 11-bromo-1-undecanol, 13-bromo-1-tridecanol or 16-bromo-1-hexadecanol. Preferred alkenyl groups are derived from 7-dodecene-1-, 11-tetradecene-1-, 11-hexadecene-1- or oleyl p-toluenesulfonate. These groups may be coupled to the linker via an ether linkage.

In a further preferred embodiment R_1, R_2 and R_3 are acyl groups and have a carbon chain length of 3-24 carbon atoms saturated or unsaturated. These groups may be coupled to the linker by ester linkages involving the acyl group.

In yet a further preferred embodiment of the present invention R_1, R_2 and R_3 are the same, and are preferably cholesterol or acyl derivatives of fatty acids including the group consisting of palmitate, myristate, laurate, caprate and oleate.

While the compound is depicted as using tromethamine to couple the spacer (and through this the nucleic acid binding domain and thence the nucleic acid itself) to 1-3 lipophilic groups, it will be apparent to those skilled in the art that a similar coupling could also be effected through an ethanolamine derivative where it is desired to use only one acyl derivative of fatty acids.

Accordingly, in a third aspect the present invention consists in a method for introducing nucleic acid into a cell comprising exposing the cell to a compound having the formula:



in which:

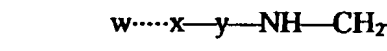
w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_5 is alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl group or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl group optionally being an acyl group having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that R_5 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

In a fourth aspect the present invention consists in a compound for use in introducing nucleic acid into a cell, the compound having the formula:



in which:

w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_5 is alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl group or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl group optionally being an acyl group having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that R_5 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

The method and the compounds of the present invention may be used for the delivery of nucleic acids including DNA, RNA, oligonucleotides (either wholly DNA or RNA or chimaeras thereof) and modified oligonucleotides into eucaryotic cells in culture including established cell lines of animal or plant origin and primary cells of animal or plant origin. With regard to delivery to plant cells and mammalian cell lines, reference is made to EPO424688, the disclosure of which is incorporated herein by reference. As plants can be regenerated from protoplasts in culture the method also provides for the production of whole plants containing the delivered nucleic acids. It can also be used to introduce such nucleic acids into whole animals. The introduction into a mammalian host may be by any of several routes including but not limited to intravenous or intraperitoneal injection, intratracheally, intrathecally, parenterally, intraarticularly, intramuscularly, etc.

The method of the present invention is generally envisaged to involve the application of the composition in an essentially aqueous mixture to the surface of the cells of interest. However, in the case of whole organisms it may be necessary to apply the composition in an essentially non-aqueous form, by localised or systemic injection, topically or by inhalation.

The lipid delivery agents can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) or related structures. To prepare LUV and MLV aqueous material is mixed with the dry lipid film and vortexed rather than sonicated. Extrusion of these mixtures through sized polycarbonate filters can produce delivery particles of more uniform size. SUVs can be prepared by

extended sonication of MLVs so producing a homogeneous population of unilamellar delivery particles. Commonly used methods for making lipid delivery particles include ether injection (Deamer, D. and Bangham, A., Biochim. Biophys. Acta (1976) **443**: 629; Ostro, et al., Biochem. Biophys. Res. Commun.(1977) **76**: 836; Fraley, et al., Proc. Natl. Acad. Sci. USA (1979) **76**: 3348); Ca^{2+}-EDTA chelation (Papahadjopoulos, et al., Biochim. Biophys. Acta (1975) **394**: 483; Wilson, et al., Cell (1979) **17**; 77) detergent dialysis (Enoch, H. and Strittmatter. P., Proc Natl. Acad Sci. USA (1979) **76**: 145 and reverse phase evaporation (REV) (Fraley, et al., J. Biol Chem. (1980) **225**: 10431; Szoka, F. and Papahadjopoulos, D., Proc. Natl. Acad. Sci. USA (1978) **75**: 145; Schaefer-Ridder, et al., Science (1982) **215**: 166). Other methods for preparing vesicular structures formed from cationic peptide/fatty acyl conjugates and suitable for transfection are also described in PCT/AU95/00505 which is incorporated herein by reference.

To enable the formation of cationic liposomes under appropriate conditions it may be necessary to include the addition of neutral lipids. It is believed that formulation of the invention into liposomes by standard methods with a neutral lipid such as phosphatidylcholine, phosphatidylethanolamine, dioleoylphosphatidylethanolamine (DOPE), DOPC (DOP choline) or cholesterol will increase the capacity of the invention to facilitate delivery of compounds into cells. This is particularly likely where the number of lipophilic groups of the invention is 2. Methods for formulating liposomes are described, for example, in Felgner, P.L. et al.(1987) Proc. Natl. Acad. Sci. (USA) **84**: 7413-7417; Yago, K et al. (1993) Biochem. Biophys. Res. Comm. **196**: 1042-1048 and Campbell, M.J.(1995) Biotechniques **18**:1027.

A major application of this invention would be as a gene therapy delivery agent where it would be expected to facilitate nucleic acid uptake by cells either *ex vivo* (with subsequent return of the genetically manipulated cells to the body) or *in vivo* (with direct uptake of nucleic acid by the tissues of a patient). Further information regarding gene therapy may be found in Nabel et al., (1993) Proc. Natl. Acad. Sci. USA **90**: 11307-11311, the disclosure of which is incorporated herein by reference. The invention would also be expected to find important application in the production of transgenic animals and plants, genetic immunisation (facilitating improved uptake and expression of nucleic acids encoding the desired antigen by the cells of the tissue to which the nucleic acid/delivery compound complexes are applied, with a subsequent immune response being raised to the expressed antigen) and gene replacement via homologous recombination.

Using this method a wide range of possible amphiphilic conjugates having 1-3 lipophilic groups can be formed. In order that the nature of the present invention might be more clearly understood, example syntheses of lead compounds will now be described in addition to the methods for assessing their transfecting activity and including an example experiment assessing the activity of a lead compound.

Description of Figures:

Figure 1.

This figure summarises the synthesis of the BOC protected spermine intermediate that was used to provide the DNA binding domain of the lead compound SpSucTL3. This synthesis is described in detail in the text.

Figure 2.

This figure summarises the synthesis of the OSU-Suc-TL3 building block as described in detail the text. This compound , when coupled with the protected spermine compound (Figure 1) produced the lead compound SpSucTL3.

Figure 3.

This figure summarises the coupling of the di-BOC-spermine compound produced as outlined in Figure 1 to the activated ester of Suc-TL3 produced as outlined in Figure 2 that resulted in the production of the lead compound SpSucTL3. This coupling is described in detail in the text.

Figure 4.

This figure summarises the synthesis of the Suc-TL3 phosphoramidite that could be used in the oligonucleotide synthesiser to produce a transfection reagent in which the nucleic acid binding domain was itself a nucleic acid. This synthesis is described in detail in the text.

Figure 5.

This figure depicts the major features of the pCIβ-Gal plasmid that was used as the reporter gene construct in the transfection experiments described in the examples. CMV IE is the immediate early promoter from human cytomegalovirus, β-Gal is the E.coli β-galactosidase gene, SVPA is the polyadenylation signal from SV40, Amp^R denotes the gene encoding resistance to the antibiotic Ampicillin. The peaked structure represents the site of a composite intron included in the pCI vector to enhance transgene expression. The approximate positions of a number of restriction enzyme sites are indicated. The positions of the Xho I and Xba I sites used to clone the β-galactosidase gene into the pCI vector are indicated in bold and underlined. The figure is not drawn to scale.

Figure 6

This figure shows the peak transfection level achieved with our lead compound SpSucTL3 and compares this to the peak transfection level achieved with the commercial reagent lipofectamine (panel B). The relative viability of cells treated under these same conditions are shown in panel A. pCIβ-Gal was transfected into cells using both reagents in the microtitre tray assay as described in the Methods an Materials section of **Assessment of Transfection Properties,** Transfection, example 4. The data in each panel represent the mean values obtained from two independent experiments. Error bars in all cases display the standard error of the mean.

Chemical Synthesis

Abbreviations used:-

BOC = tert-Butyloxycarbonyl
DCCD = Dicyclohexylcarbodiimide
DCM = Dichloromethane
DIEA = Diisopropylethylamine
DMAP = Dimethylaminopyridine
DMF = Dimethylformamide
HOSU = N-Hydroxysuccinimide
HPLC = High performance liquid chromatography
Suc = succinic acid
TFA = Trifluoroacetic acid
THF = Tetrahydrofuran
Tris= 2-Amino-2-hydroxy-methyl-1,3 propanediol
Z = Bezyloxycarbonyl

Methods used:

Thin Layer Chromatography (TLC)

Thin layer chromatography was performed on Alufolein Silica gel 60 F_{254} plates (Merck) in the following solvent systems; DCM/MeOH = 97/3 (R_f^1), chloroform/MeOH =65/35 (R_f^2), DCM/MeOH/DIEA = 98/2/1 (R_f^3)

High Performance Liquid Chromatography (HPLC)

Analytical HPLC was carried out on Millipore Waters HPLC equipment (Waters Chromatography Division of Millipore, Milford, MA), comprising of a 6000A series solvent delivery system with an automated gradient controller and Model 746

Data Module, The chromatography was carried out with a NOVAPAK^{TM} C_{18} reverse phase column (100x8 mm). The peptides and the peptide-Tris conjugates were analysed on a linear gradient elution from 24 to 80% acetonitrile with 0.1% (TFA within 5 min at a flow rate of 2 ml/min (System A). Detection was carried out at 260 nm using a Waters Lambda Max 480; (R_t^A).

The lipopeptide conjugates were analysed on a C_{18} column with a linear gradient from 40% water, 50% acetonitrile and 10% THF to 50% acetonitrile, 50% THF with 0.1% TFA within 5 min at a flow rate of 2 ml/min (System B); (R_t^B).

Preparative HPLC

Separation were carried out on a Millipore Waters DeltaPrep 4000 HPLC using a PrePak C_{18} reverse phase column (100x40mm) eluted with a linear gradient with the same elution buffer systems as mentioned above for the analytical HPLC at a flow rate of 20 ml/min.

Nuclear Magnetic Resonance (NMR)

NMR spectra were recorded with a 200 MHz Brucker spectrophotometer.

Example 1

METHOD FOR PREPARATION OF SPERMINE-SUC-TRIS-TRILAURATE

Preparation of N^4-N^9-di-BOC-Spermine

This compound was synthesised in 3 steps as reported by Goodnow et al..(1990) Tetrahedron **46**: 463267-3286 with some modification as shown in Figure 1 and described below.

Step 1: Preparation of N, N'(diaminobutyl)-diethylnitrile (1)

Acrylonitrile (3.9 g, 73.5 mmol) was added to diaminobutane (4.3 g, 48.8 g) in 5 ml of MeOH at 0° C and the reaction mixture was stirred for 5 h. The solvent was evaporated to dryness and the oily residue was purified by silica gel flash chromatography eluting with chloroform/MeOH with an increasing ratio of MeOH to obtain 2.1 g of the title compound (R_f^2=0.4). The structure of this compound was confirmed by ^{1}HNMR.

Step 2: Preparation of N, N'-di-BOC-(diaminobutyl)-diethylnitrile (2)

Compound 1 (2 g, 10.3 mmol) was dissolved in 15 ml of DCM. BOC-anhydride (4.5 g , 20.6 mmol) was added to it and the reaction mixture was stirred at room temperature for 3 h. The reaction mixture was then washed with sodium bicarbonate and water and the organic phase was dried over $MgSO_4$ and then evaporated to dryness to obtain 4.2 g of the title compound ((R_f^1=0.84). The structure of this compound was confirmed by ^{1}HNMR.

Step 3: Preparation of N4, N9-di-BOC-Spermine (3)

To 560 mg of compound 2 (1.4 mmol) in 10 ml of diethylether, Li Al H_4 (0.19 g, 5 ml of 1 M solution in diethylether) was added at 0° C and the reaction mixture was stirred at 0° C for 1 h. The excess Li Al H_4 was quenched by adding 3 ml of 1 N NaOH at 0° C. The precipitate was filtered and the filtrate was washed with water. The organic layer was dried over $MgSO_4$ and evaporated to dryness to obtain 270 mg of the title compound. The structure of this compound was confirmed by ^{1}HNMR.

Preparation of OSU-Suc-Tris-trilaurate

This 5 step synthesis is outlined in Figure 2.

Step1: Preparation of Z-Tris (4)

To a solution of Z-OSU (4.98 g, 20 mmol) in 30 ml of acetonitrile, Tris (6.6 g, 60 mmol) in 20 ml of water was added. The reaction mixture was stirred for 1 h to obtain 82% of Z-Tris confirmed by HPLC (R_t^A: 4.24). The solvents were evaporated to dryness, the residue was redissolved in ethyl acetate and the excess Tris was washed away with water. The organic layer was dried over sodium sulfate and evaporated to dryness to give 3.2 g of pure Z-Tris confirmed by ^{1}HNMR.

Step 2: Preparation of Z-Tris trilaurate (5)

Z-Tris (2.8 g, 10.9 mmol) was dissolved in 6 ml of DMF and 30 ml of DCM. DCCD (6.57 g, 3.27 mmol) and lauric acid (6.56 g, 32.7 mmol) and a catalytic amount of DMAP was added to the reaction mixture and it was stirred overnight. HPLC after 16 h of reaction showed formation of the title compound in 49% yield along with 41% of dilaurate and 10% of monolaurate. The solvents were evaporated to dryness and the residue was redissolved in DCM. The precipitate of dicyclohexylurea (DCU) was filtered and the filtrate washed with sodium bicarbonate (5%) and water. Preparative HPLC of the mixture yielded high purity of the title compound (3.5 g, R_t^B: 9.26).

Step 3: Preparation of Tris-trilaurate (6)

Z-Tris-trilaurate (3.4 g, mmol) was dissolved in DCM/methanol (50/50, 60 ml) and hydrogenated for 3 h at 40 psi in a parr hydogenator using 10% palladium/carbon to remove the Z group. Palladium/carbon was filtered and the filtrate was evaporated to dryness to obtain 2.2 g of the title compound. The removal of the Z group was confirmed by HPLC and ^{1}HNMR spectroscopy.

Step 4: Preparation of Suc-Tris-trilaurate (7)

Tris-trilaurate (1.5 g, 2.24 mmol) was dissolved in 20 ml of DCM. Succinic anhydride (0.45 g, 4.49 mmol) and DIEA (382 ml , 2.24 mmol) were added to the reaction mixture which was then stirred overnight at room temperature. The reaction was followed by TLC (R_f^1=0.42). The excess succinic anhydride was washed away

with water. DCM was evaporated to dryness to obtain 1.9 g of the title compound confirmed by ^{1}HNMR

Step 5: Preparation of OSU-Suc-Tris-trilaurate (8)

Suc-tris-trilaurate (1g, 1.3 mmol) was dissolved in 8 ml of DCM. DCCD (0.268, 1.30 mmol) and HOSU (0.224, 1.95 mmol) were added and the reaction mixture was stirred for 16 h. TLC of the reaction mixture showed quantitative formation of the title compound (R_f^1=0.50). DCU was filtered and the filtrate was evaporated to dryness and used in the next coupling without further purification.

Preparation of N^1-(N^{12}-Z, N^4, N^9-di-BOC-Spermine)Suc-Tris-trilaurate (10)

To a solution of compound 3 (67 mg, 0.168 mmol) in 5 ml of DCM, compound 8 (0.168 mmol) in 8 ml of DCM was added and the pH of the reaction mixture was adjusted to 8 by adding DIEA. The reaction mixture was stirred overnight to obtain compound 9. Z-OSU (80 mg, 0.32 mmol) was added to the above reaction mixture and it was stirred for additional 2 h to obtain the title compound 10. Preparative HPLC of the above mixture yielded 12 mg of pure compound 10 and the rest of the fractions were mixtures of the title product with compound 7 and compound 9. The structure of the title compound was confirmed by ^{1}HNMR.

Preparation of N^1-Spermine-Suc-Tris-trilaurate (11)

Compound 10 was dissolved in 3 ml (DCM/MeOH=50/50) and hydrogenated for 2h at 40 psi in a parr hydogenator using 10% palladium/carbon to remove the Z group. The removal of the Z group was confirmed by HPLC. Palladium/carbon was then filtered and the filtrate was evaporated to dryness. The removal of Z was confirmed by ^{1}HNMR spectroscopy. The residue was redissolved in 0.5 ml of DCM and cooled to 0° C. TFA (0.5 ml) was added and the reaction mixture was stirred for 10 min at 0° C and 30 min at room temperature. TFA and DCM were evaporated to dryness and the residue was lyophilised to obtain of the title compound.

Other analogs with different lengths of the polyamine, different kinds of spacer and different types of fatty acyl groups can be synthesised according to the method described above with slight modification as required.

Example 2

METHOD FOR PREPARATION OF DNA-TRIS-TRILAURATE CONJUGATES WITH A PHOSPHORAMIDITE LINKER IN THE 5' TERMINUS OF THE DNA.

This synthesis is summarised in Figure 4.

Preparation of OSU-Suc-Tris-trilaurate

This compound was synthesised as shown in example 1.

Preparation of Hex-Suc-Tris-trilaurate (12)

This synthesis is summarised in Figure 3.

To a solution of 1.3 mmol of OSU-Suc-Tris-trilaurate in 20 ml of DCM, 6-amino-hexanol (152 mg, 1.3 mmol) was added and the reaction mixture was stirred overnight. The solvents were evaporated to dryness and the residue redissolved in DCM and purified with a silica gel flash chromatography column, eluting with DCM/MeOH using an increasing ratio of MeOH. 400 mg of the title compound (R_f^3: 0.28) was obtained and the structure was confirmed by ^{1}HNMR.

Preparation of Phosphoramidite-Hex-Suc-Tris-trilaurate (13)

Compound 12 (160 mg, 0.189 mmol) was dissolved in 3 ml of dry THF and chloro diisopropyl cyanoethoxy phosphine (89 mg, 0.380 mmol) and DIEA (96 µl) were both added to the reaction mixture. The mixture was stirred for 2 h under nitrogen. The solvent was then evaporated to dryness and the residue was purified by silica gel flash chromatography eluting with DCM/MeOH/DIEA with an increasing ratio of MeOH. 98 mg of the title compound (R_f^3: 0.3) was obtained and the structure of this compound was confirmed by ^{1}HNMR.

Preparation of DNA-Tris-laurate conjugates

The phosphoramidite-Hex-Suc-Tris-trilaurate was dissolved in DCM and used like a phosphoramidite nucleoside and coupled to the 5' hydroxyl terminus of the support bound DNA in the final coupling cycle. Ammonia cleavage and deprotection can give the fatty acid-DNA conjugate in solution.

Example 3

METHOD FOR PREPARATION OF DNA-TRIS-MONO, DI, AND TRILAURATE CONJUGATES WITH AN AMINOLINKER EITHER IN THE 3' OR 5' TERMINUS

Preparation of thioglycolate-mono, di, and trilaurate

Thioglycolic acid can be activated with HOSU in the presence of DCC. The activated ester can then be coupled to Tris in a DMF solution. It can then be purified by extraction with ethyl acetate. This product can be acylated with lauric acid in the presence of DCC to give a mixture of the title products. The three compounds can then be separated by silica gel chromatography with elution by organic solvents.

Preparation of DNA-iodo-acetyl

Iodoacetic acid can be activated with HOSU in the presence of DCC. This activated ester can then be coupled to the aminolinker in the 3' or 5' terminus of the DNA; an aminolinker group can be attached to DNA either on 3' or 5' end by commercially available aminolink reagent. The product can then be purified on an anion-exchange column.

Preparation of DNA-acetyl-glycolate-Tris-laurate

The DNA-iodo-acetyl can be coupled specifically to the thiol group of the thioglycolate-laurate immediately after preparing the DNA iodo acetyl. The product can then be purified by reverse phase HPLC.

Assessment of Transfection Properties

Abbreviations used:

β-Gal = β-galactosidase (from E. coli)

CAT = Chloramphenicol Acetyl Transferase

CHO = Chinese Hamster Ovary cells

Cos1 = African Green Monkey kidney cells carrying the large T antigen from SV40 virus.

DME = Dulbecco's Modified Eagles medium

DMSO = Dimethylsulphoxide

DPBS = Dulbecco's Phosphate Buffered Saline

EMEM = Earl's Modified Eagles Medium

FCS = Foetal Calf Serum

h = hours

lac Z = gene encoding E. coli β-galactosidase

min = minutes

MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt

PBS = Phosphate Buffered Saline

PMS = Phenazine methosulphate

PGK = Phosphoglycerate Kinase

X-Gal = 5-Bromo-4-chloro-3-indolyl-β-D-glactopyranoside

Example 4

TRANSFECTION OF CULTURED CELLS BY THE LEAD COMPOUND SpSucTL3.

Methods and Materials used:

Compounds and Nucleic Acids

A lead compound, SpSucTL3 (spermine-succinic acid spacer-Tris-trilaurate), was synthesised as described above. The compound was dissolved at 20 mM in ethanol and diluted with an equal volume of sterile water to provide a 10 mM stock solution in 50% ethanol. This 10 mM stock solution was further diluted in sterile water with vortexing to produce a 2 mM working stock. The commercially available cationic lipid transfection reagent lipofectamine (GIBCO/BRL) was used as a positive control.

To test the transfection properties of the lead compound SpSucTL3, whole uncut pCIβ-Gal plasmid which carries the E.coli β-Galactosidase reporter gene under the control of the cytomegalovirus immediate early promoter was used. To generate this reporter gene expression cassette the E.coli β-Galactosidase gene from pSVGAL (Sleigh, M.J. and Lockett, T.J. (1985). EMBO J. 4: 3831-3837) was first cloned as a Hind III-Bam HI fragment into Bluescript SK+ (Clontech) to produce Bst β-Gal. The reporter gene was then excised from Bst β-Gal as a Xho I-Xba I fragment and cloned into Xho I-Xba I cut pCI (Clontech) to produce the expression plasmid shown in Figure 5.

Transfection protocol

Transfection efficiency was determined by measuring the level of reporter gene product (β-Gal activity) present in cells 48 h after introducing the reporter gene cassette into the cells. To achieve this each well in an 8 x 9 array of wells in a microtitre dish was seeded with 1 x 10^4 CHO cells in EMEM, 10% FCS and allowed to adhere overnight. The next day, in a new microtitre tray, a DNA dilution plate was set up by placing 250 µl of EMEM containing 4.0 µg of pCIβ-Gal plasmid in each of the top nine wells of the plate. EMEM (125 µl) was added to the seven rows of nine wells immediately below the DNA containing wells. The DNA was then serially diluted in two fold steps through the seven rows of medium-containing wells resulting in a plate with eight rows of wells in which all wells of any given row carried an identical amount of DNA. In an analogous way, a transfection agent dilution plate was also prepared. To eight wells of the left most column of a new microtitre plate was added 120 µl of EMEM containing the transfection reagent (SpSucTL3 or lipofectamine) at 168 µM. To eight wells of the adjacent eight columns of wells was added 60 µl of DME. The transfection reagent was then be serially diluted in two-fold steps through seven of the

columns of medium-containing wells resulting in eight columns of wells in which all wells of any given column contained identical concentrations of the transfection reagent to be tested and a final column containing no reagent. The DNA and transfection reagent dilution matrices were then superimposed by transferring 60 µl of DNA containing medium from the wells of each row of the DNA dilution plate to the corresponding row of the transfection reagent dilution plate. This plate was optionally shaken for 1 min. After mixing the plate carrying the combined reagent and DNA was allowed to stand at room temperature for 10 min to facilitate formation of the transfection complex prior to application to cells. Cells were washed once with EMEM then the transfection matrix was transferred to the cell plate, 100 µl of the appropriate DNA/reagent mix per well. Plates were incubated for 4 h at 37°C, 95% humidity in 5% CO_2. EMEM, 10% FCS (100 µl) was then be added to each well and the plates incubated overnight. Next morning the medium was replaced with fresh EMEM, 10% FCS and the plates returned to the incubator for the reminder of the 48 h expression time.

Analysis of toxicity

As a part of each transfection study, toxicity of the test compound in combination with nucleic acid was tested using the MTS assay prior to assaying for reporter gene expression. MTS (Promega)(2 mg/ml in DPBS) and PMS (Promega) (0.92 mg/ml in DPBS) were mixed fresh at a ratio of 20:1 and 20 µl of this mix per 100 µl of culture medium was added to each test and control well of each microtitre plate . Cultures were then incubated for a further 0.5-2 h under normal conditions (37°C, 95% humidity and 5% CO_2). Cell viability was then determined by calculating the difference between the A_{490} (test) and A_{655} (reference) readings for all samples and controls using a plate reader, knowing the relationship between number of viable cells and the magnitude of this difference.

Measurement of transfection activity via reporter gene expression.

MTS-containing medium was first removed and the cells washed 1x with PBS. For the β-Gal assay, the washed cells in each well were lysed by the addition of 50 µl of lysis buffer (0.1% Triton X-100, 250 mM pH 8.0). Plates were sealed, frozen at -70°C, thawed and 50 µl of PBS, 0.5% BSA added to each well. To quantify β-Gal activity, 150 µl of substrate in buffer (1 mg/ml chlorophenol red galactopyranoside (Boehringer Mannheim) in 60 mM sodium phosphate buffer pH 8.0, 1 mM $MgSO_4$, 10 mM KCl, 50 mM 2-mercaptoethanol) was added to each well and colour allowed to develop at room temperature for 1 min to 24 h. Activity was determined by reading the A_{570} for each well and comparing it to those produced by known set of β-Gal standards.

The results obtained are shown in Figure 6. Figure 6A shows that both SpSucTL3 and lipofectamine show similar levels of toxicity in those wells in which they show maximum transfection activity (21 μM lipid, 0.25 μg plasmid for Lipofectamine; 21 μM lipid, 0.5 μg plasmid for SpSucTL3). Figure 6B shows that the SpSucTL3 can effectively introduce reporter gene constructs into cultured cells. While this activity is slightly lower than that observed with lipofectamine, it will be apparent to those skilled in the art that SpSucTL3 represents a lead compound for a new family of related compounds. By making systematic alterations to the length, number and chemical nature of the hydrophobic domains; the length and nature of the spacer region and the size, charge, nature and linkage of the nucleic acid binding domain and/or by formulation with neutral lipids it will be possible to synthesise reagents with greatly improved nucleic acid delivery properties without departing from the essential features of the molecular designs described in this application. It may be possible to further enhance the transfection properties of these reagents by including polycations with the nucleic acid and reagent at the time of formation of the transfection complexes as has previously been described for a number of cationic liposomes (Gao, X. and Huang, L.(1996) Biochemistry **35**: 1027-1036).

While the exact mechanism by which SpSucTL3 enhances transfection of cells is not known, since it is a cationic lipid, we assume that it interacts with the anionic nucleic acids through a charge interaction providing a lipid coating that will facilitate their cellular uptake as hypothesised by Gershon et al. (Gershon, H. et al. (1993) Biochemistry **32**: 7143).

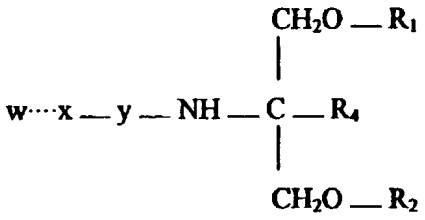
In addition to delivering nucleic acids containing gene expression cassettes the transfection reagents of this invention could also be used to deliver oligonucleotides (whether composed of RNA, DNA, protected nucleotides or combinations of these) into cells. The efficiency with which the transfection reagents can deliver such nucleic acids to cells could be determined by labelling the oligonucleotides by any of a number of methods well known in the art including, but not limited to, using radioactivity, fluorescent tags, biotin and digoxigenin. By way of example, where the proportion of cells transfected with oligonucleotide using a particular reagent is to be measured using fluoresceinated oligonucleotides, the following approach could be used. Briefly, 8 x 10^4 Cos1 cells (5 x 10^4 CHO cells) would be seeded onto coverslips in the wells of a 24 well plate in DME (for Cos1 cells) or EMEM (for CHO cells), 10% FCS and incubated at 37^{o}C in 5% CO_2/air to allow cell attachment. A Fluoresceinated oligonucleotide (1-20 μM in 250 μl of medium) would be mixed with transfection reagent (SpSucTL3 or Lipofectamine, 0.25, 0.5 or 1 μl of a 2 mM stock in 250 μl of

medium) and allowed to stand at room temperature for 15 min. Cells would then be washed 1x with serum free medium and 500 µl of the different transfection mixes would be added to the wells. Plates would then be incubated at 37°C, 95% humidity in 5% CO_2 for 4 h. After incubation the medium would be removed and the cells washed 1x with PBS. Cover slips would be removed from the wells, mounted in PBS and examined by confocal scanning laser microscopy to score the proportion of cells containing the fluorescent label.

It will be appreciated by persons of moderate skill in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit and scope of the invention as broadly described.

Claims:-

1. A method for introducing nucleic acid into a cell comprising exposing the cell to a compound having the formula:



in which:

w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_4 is H or halogen or CH_2O - R_3; and R_1, R_2 and R_3 are the same or different and are either hydrogen, methyl, ethyl, alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl groups or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl groups, optionally being an acyl group derived from a fatty acid having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that at least one of R_1, R_2 or R_3 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

2. A method as claimed in claim 1 in which y is present.

3. A method as claimed in claim 1 in which the nucleic acid is DNA, RNA or oligonucleotides of either DNA or RNA, modified oligonuleotides or a combination thereof.

4. A method as claimed in claim 1 in which R_1, R_2 and R_3 are the same.

5. A method as claimed in claim 1 in which R_1, R_2 and/or R_3 are cholesterol or acyl derivatives of fatty acids selected from the group consisting of palmitate, myristate, laurate, caprate and oleate.

6. A method as claimed in claim 5 in which R_1, R_2 and/or R_3 are acyl derivatives of myristate or laurate.

7. A method as claimed in any claim 1 in which the cells are animal cells.

8. A method as claimed in claim 1 in which the cells are plant cells.

9. A method as claimed in claim 7 in which the method is conducted *in vitro*.

10. A method as claimed in claim 7 in which the method is conducted *in vivo*.

11. A method as claimed in claim 10 in which the compound is administered topically, intravenously, intramuscularly, by inhalation, injection, orally or by suppository.

12. A method as claimed in claim 1 in which the compound is present in a liposome or mixed with another lipid.

13. A method as claimed in claim 1 in which the compound contains a spacer group "y" having a chain length equivalent to 3 to 17 carbon atoms.

14. A method as claimed in claim 13 in which "y" is amino butyric, amino caproic, amino caprylic or amino undecanoic acid or a dipeptide of amino caproic acid and amino undecanoic acid.

15. A method as claimed in claim 1 in which "x" has an overall positive charge and the nucleic acid is associated electrostatically.

16. A method as claimed in claim 1 in which "x" is an oligonucleotide and nucleic acid "w" is associated with "x" by base pairing or triple helix formation.

17. A method as claimed in claim 1 in which "w" is covalently attached to "x"

18. A method as claimed in claim 1 in which "w" is associated to "x" by hydrogen bonding.

19. A method for introducing nucleic acid into a cell comprising exposing the cell to a compound having the formula:

in which:

w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_5 is alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl group or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl group optionally being an acyl group having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that R_5 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

20. A method as claimed in claim 19 in "y" is present.

21. A method as claimed in claim 19 in which the nucleic acid is DNA, RNA or oligonucleotides of either DNA or RNA, modified oligonucleotides or a combination thereof.

22. A method as claimed in claim 19 in which R_5 is cholesterol or an acyl derivative of a fatty acids selected from the group consisting of palmitate, myristate, laurate, caprate and oleate.

23. A method as claimed in claim 22 in which R_5 is an acyl derivative of myristate or laurate.

24. A method as claimed in claim 19 in which the cells are animal cells.

25. A method as claimed in claim 19 in which the cells are plant cells.

26. A method as claimed in claim 24 in which the method is conducted *in vitro*.

27. A method as claimed in claim 24 in which the method is conducted *in vivo*.

28. A method as claimed in claim 27 in which the compound is administered topically, intravenously, intramuscularly, by inhalation, injection, orally or by suppository.

29. A method as claimed in claim 19 in which the compound is present in a liposome or mixed with another lipid.

30. A method as claimed in claim 19 in which the spacer group "y" has a chain length equivalent to 3 to 17 carbon atoms.

31. A method as claimed in claim 30 in which "y" is amino butyric, amino caproic, amino caprylic or amino undecanoic acid or a dipeptide of amino caproic acid and amino undecanoic acid.

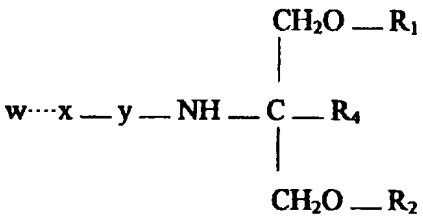
32. A method as claimed in claim 19 in which "x" has an overall positive charge.

33. A method as claimed in claim 19 in which "x" is an oligonucleotide and nucleic acid "w" is associated with "x" by base pairing or triple helix formation

34. A method as claimed in claim 19 in which "w" is covalently attached to "x".

35. A method as claimed in claim 19 in which "w" is associated to "x" by hydrogen bonding.

36. A compound for use in introducing nucleic acid into a cell, the compound having the formula



in which:

w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_4 is H or halogen or CH_2O - R_3; and R_1, R_2 and R_3 are the same or different and are either hydrogen, methyl, ethyl, alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl groups or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl groups, optionally being an acyl group derived from a fatty acid having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that at least one of R_1, R_2 or R_3 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

37. A compound as claimed in claim 36 in which "y" is present.

38. A compound as claimed in claim 36 in which "w" is DNA, RNA or oligonucleotides of either DNA or RNA, modified oligonucleotides or a combination thereof.

39. A compound as claimed in claim 36 in which R_1, R_2 and R_3 are the same.

40. A compound as claimed in claim 36 in which R_1, R_2 and/or R_3 are cholesterol or acyl derivatives of fatty acids selected from the group consisting of palmitate, myristate, laurate, caprate and oleate.

41. A compound as claimed in claim 40 in which R_1, R_2 and/or R_3 are acyl derivatives of myristate or laurate.

42. A compound as claimed in claim 36 in which the compound is present in a liposome or mixed with another lipid.

43. A compound as claimed in claim 36 in which the compound contains a spacer group "y" having a chain length equivalent to 3 to 17 carbon atoms.

44. A compound as claimed in claim 43 in which "y" is amino butyric, amino caproic, amino caprylic or amino undecanoic acid or a dipeptide of amino caproic acid and amino undecanoic acid.

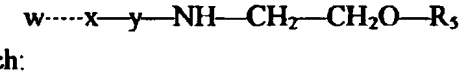
45. A compound as claimed in claim 36 in which "x" has an overall positive charge.

46. A compound as claimed in claim 36 in which "x" is an oligonucleotide and nucleic acid "w" is associated with "x" by base pairing or triple helix formation.

47. A compound as claimed in claim 36 in which "w" is covalently attached to "x".

48. A compound as claimed in claim 36 in which "w" is associated to "x" by hydrogen bonding.

49. A. compound for use in introducing nucleic acid into a cell, the compound having the formula:



in which:

w is a nucleic acid

x is a non-amino acid or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon single covalent bonds or is absent

R_5 is alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl group or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl group optionally being an acyl group having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that R_5 includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

50. A compound as claimed in claim 49 in which "y" is present.

51. A compound as claimed in claim 49 in which "w" is DNA, RNA or oligonucleotides of either DNA or RNA, modified oligonucleotides or a combination thereof.

52. A compound as claimed in claim 49 in which R_5 is cholesterol or an acyl derivative of a fatty acids selected from the group consisting of palmitate, myristate, laurate, caprate and oleate.

53. A compound as claimed in claim 52 in which R_5 is an acyl derivative of myristate or laurate.

54. A compound as claimed in claim 49 in which the compound is present in a liposome or mixed with another lipid.

55. A compound as claimed in claim 49 in which the compound contains a spacer group "y" having a chain length equivalent to 3 to 17 carbon atoms.

56. A compound as claimed in claim 55 in which "y" is amino butyric, amino caproic, amino caprylic or amino undecanoic acid or a dipeptide of amino caproic acid and amino undecanoic acid.

57. A compound as claimed in claim 49 in which "x" has an overall positive charge.

58. A compound as claimed in claim 49 in which "x" is an oligonucleotide and nucleic acid "w" is associated with "x" by base pairing or triple helix formation.

59. A compound as claimed in claim 49 in which "w" is covalently attached to "x".

60. A compound as claimed in claim 49 in which "w" is associated to "x" by hydrogen bonding.

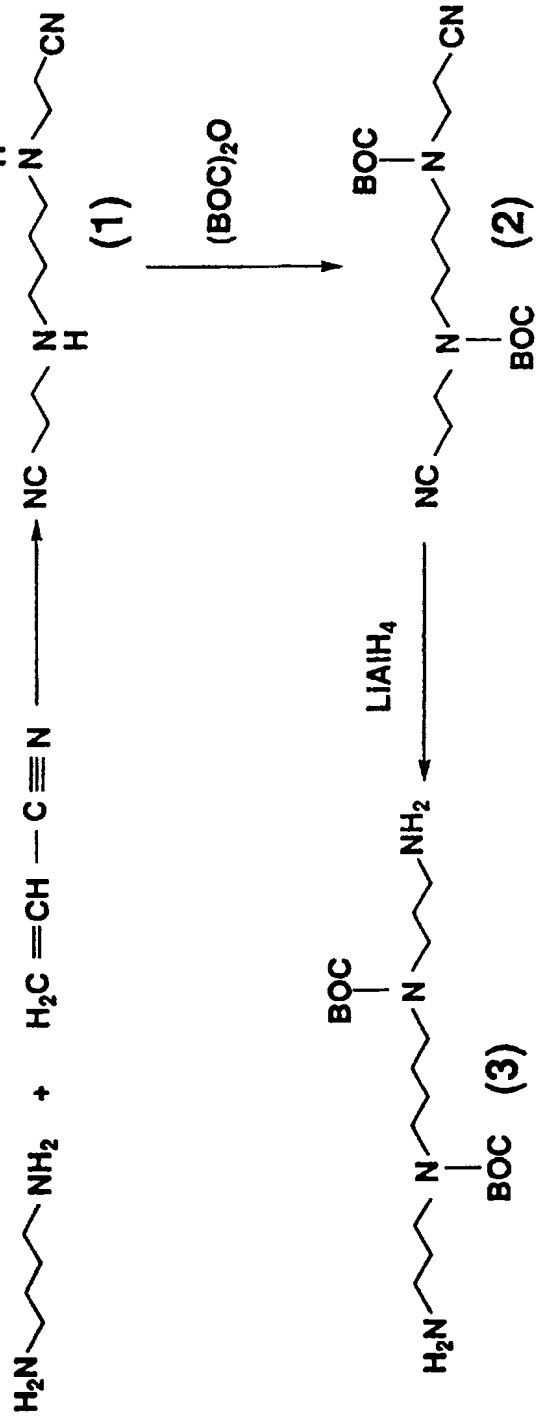


Figure 1.

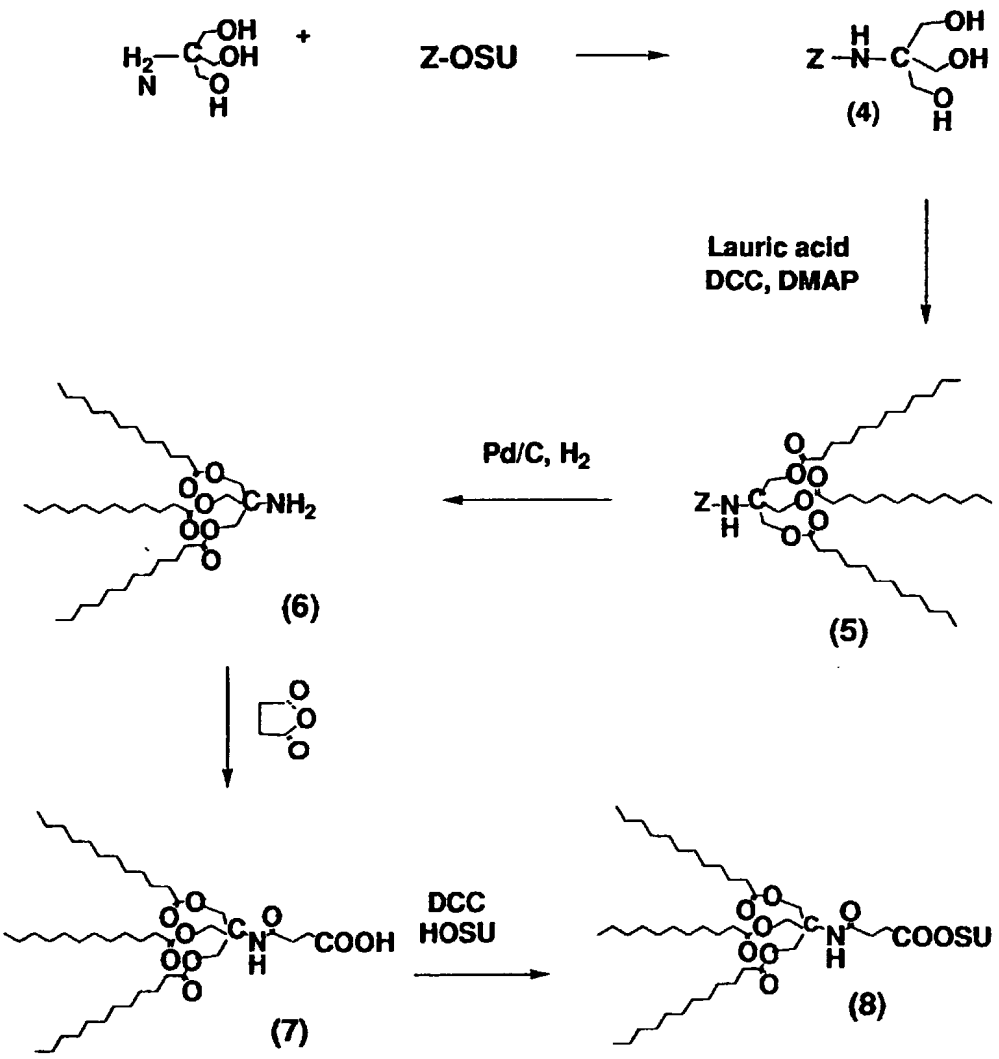


Figure 2.

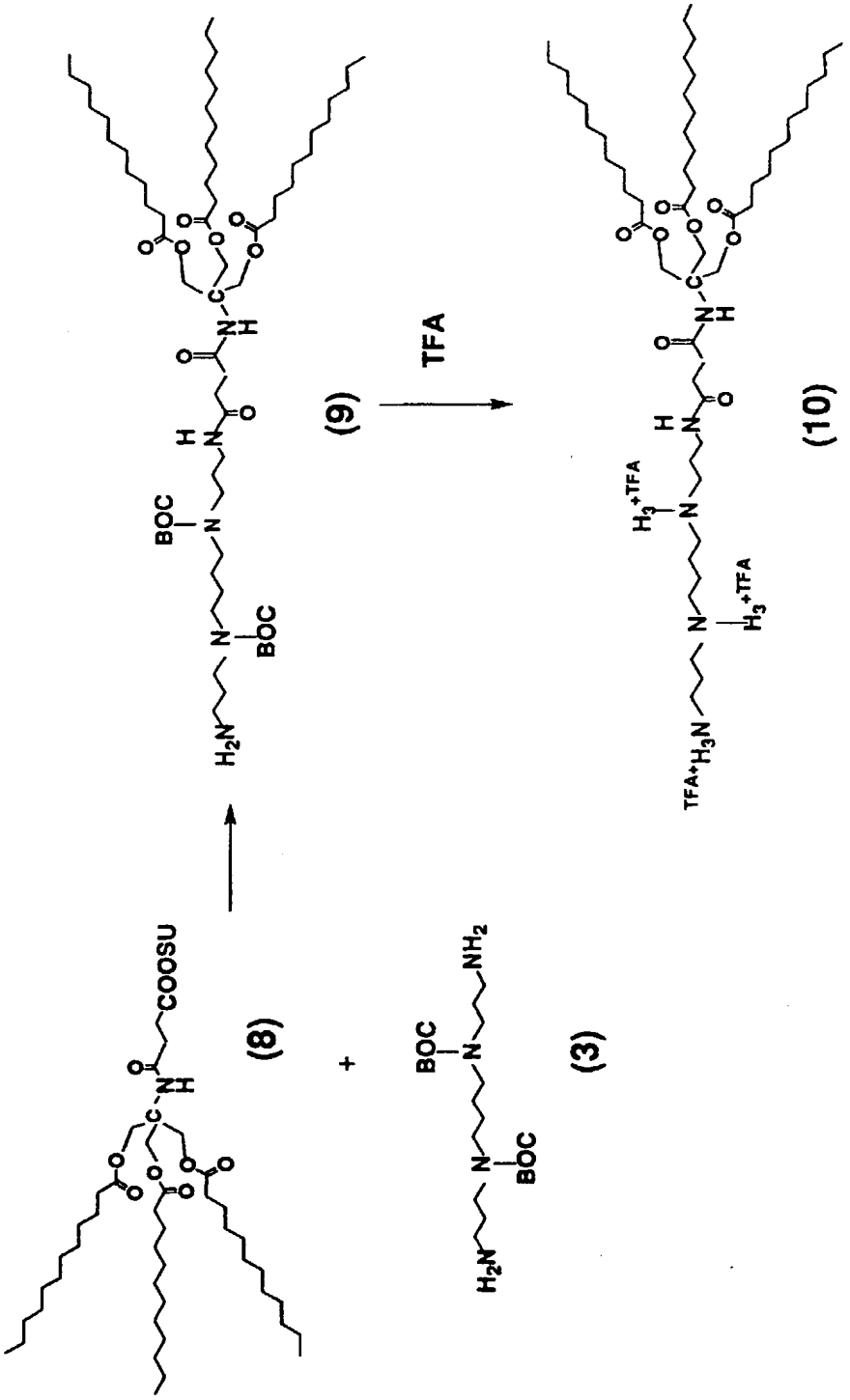


Figure 3.

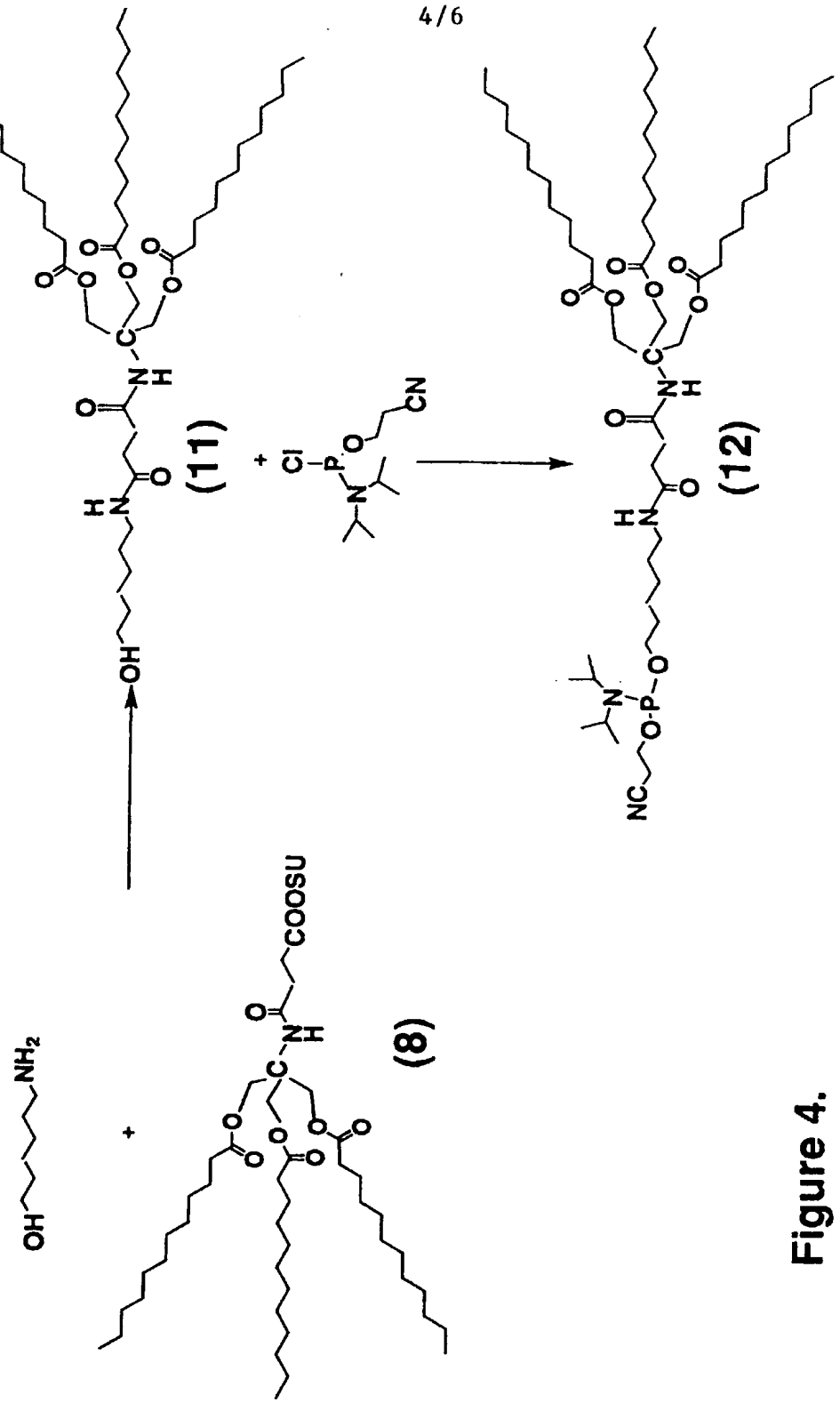


Figure 4.

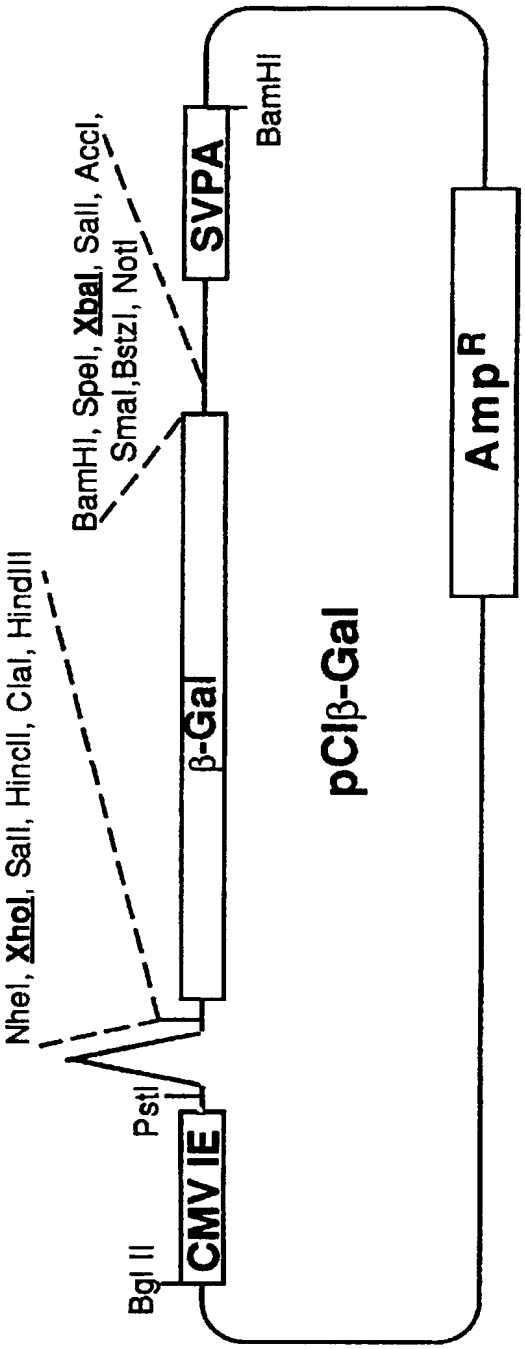
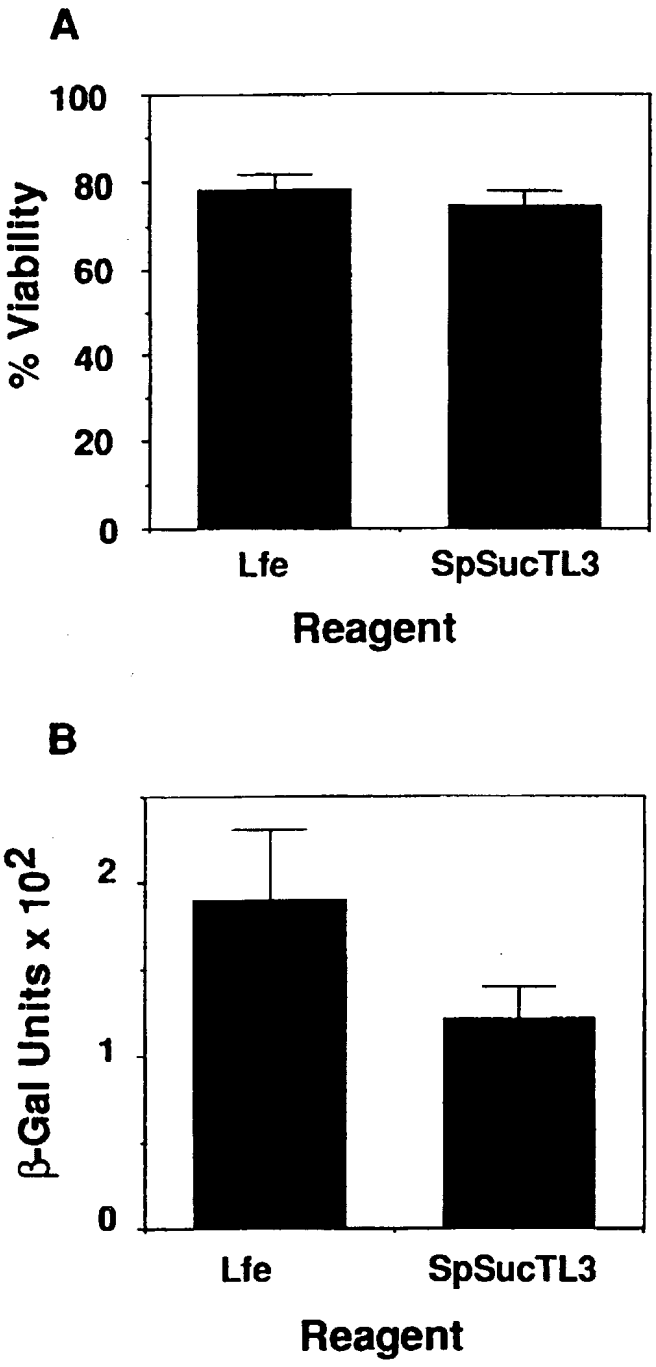

BamHI
SVPA
BamHI, SpeI, XbaI, SalI, AccI,
SmaI,BstZI, NotI
β-Gal
NheI, XhoI, SalI, HincII, ClaI, HindIII
PstI
CMV IE
Bgl II
pCIβ-Gal
Amp^R

Figure 5

Figure 6


A
100
80
60
40
20
0
% Viability
Lfe
SpSucTL3
Reagent
B
2
1
0
β-Gal Units x 10²
Lfe
SpSucTL3
Reagent

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00004

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07H 21/02, 21/04, C12N 15/88, A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: C07H-021, C12N 15/87, 15/88, A61K 48/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT
CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 96/05218 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 22 February 1996 entire document	1-60
P, X	WO 96/22303 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 25 July 1996 claims 21-32	1-60
X	WO 95/04030 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 9 February 1995 entire document	1-60

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
5 February 1997

Date of mailing of the international search report
17 FEBRUARY 1997

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00004

C (Continuation) **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91/09837 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 11 July 1991 entire document	1-60
P, X	WO 96/32102 A (PASTEUR MERIEUX SERUMS ET VACCINS) 17 October 1996 entire document	1-60
P, A	WO 96/17823 A (RHONE-POULENC RORER S.A.) 13 June 1996 entire document	1-60
A	WO 93/05162 A (THE UNIVERSITY OF TENNESSEE RESEARCH CORPORATION) 18 March 1993 entire document	1-60
A	WO 91/16024 A (VICAL, INC.) 31 October 1991 entire document	1-60
A	Behr J. et al., "Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA", *Proc. Natl. Acad. Sci. USA*, (September 1989) 86, 6982-6 entire document	1-60
A	Gao X. et al., "A novel cationic liposome reagent for efficient transfection of mammalian cells", *Biochem. Biophys. Res. Commun.* (30 August 1991) 179(1), 280-5 entire document	1-60
P, A	Vigneron J. et al., "Guanidinium-cholesterol cationic lipids: Efficient vectors for the transfection of eukaryotic cells", *Proc. Natl. Acad. Sci. USA*, (September 1996) 93, 9682-6 entire document	1-60
P, A	Moradpour D. et al. "Efficient gene transfer into mamalian cells with cholesteryl-spermidine". *Biochem. Biophys. Res. Commun.* (1996) 221(1), 82-88 entire document	1-60

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/AU 97/00004

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9605218	AU	31585/95				
WO	9622303	AU	44269/96				
WO	9504030	AU	73420/94	CA	2167818	CN	1128531
		EP	712389	FI	960504	NO	960389
WO	9109837	AU	70336/91	EP	506748	US	5583198
WO	9305162	AU	26565/92	EP	663013	US	5283185
		US	5480817				
WO	9116024	AU	78547/91	EP	523189	US	5264618
		US	5459127				
WO	9632102	AU	56517/96	FR	2732895		
WO	9617823	AU	43072/96	FR	2727679	ZA	9510326

END OF ANNEX